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The cytoplasmic tail of Herpes simplex virus glycoprotein H binds to the tegument protein VP16 in vitro and in vivo

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Abstract

During Herpes simplex virus envelopment, capsids, tegument polypeptides, and membrane proteins assemble at the site of budding and a cellular lipid bilayer becomes refashioned into a spherical envelope. Though the molecular interactions driving these events are poorly understood, several lines of evidence suggest that associations between envelope protein cytoplasmic tails and tegument polypeptides may play important roles. Consistent with this hypothesis, we show here that a fusion of the cytoplasmic tail of gH with Glutathione-*S*-Transferase binds to VP16 in a temperature-dependent manner. VP16 prepared by in vitro translation behaves in a similar fashion, demonstrating that the interaction is not dependent on other viral polypeptides. Mutational analysis of the gH tail has also enabled us to identify amino acid residues critical for VP16 binding in vitro. A fusion protein in which the gH tail is fused to the carboxy-terminus of GFP coimmunoprecipitates with VP16 in infected cells, indicating that VP16 can interact with the gH tail in vivo.

Keywords: HSV-1; gH; VP16; Envelopment; Glycoprotein; Tegument

Introduction

The Herpesvirus particle is composed of a proteinaceous, icosahedral capsid that is assembled and packaged with the double-stranded DNA genome in the nucleus of infected cells. The mature capsid is surrounded by a complex of proteins known as the tegument and is enveloped within a lipid bilayer derived from host cell membranes. This envelope also contains a number of virally encoded glycoproteins (Roizman and Pellett, 2001). While the site of final tegument and envelope assembly has been controversial (Enquist et al., 1998; Mettenleiter, 2002), recent genetic, biochemical, and ultrastructural data suggest that Herpes simplex virus type 1 (HSV-1) capsids acquire their final envelope and tegument layer by budding into the *trans*-Golgi network (TGN) or endosomes (Browne et al., 1996a; Brunetti et al.,1998; Whiteley et al.,1999; Harley et al., 2001; Skepper et al., 2001; McMillan and Johnson, 2001; Miranda-Saksena et al., 2002).

In HSV-1, at least 15 different viral polypeptides have been identified as components of the tegument, and more than 10 different glycoproteins are associated with the envelope (Spear, 1994; Roizman and Knipe, 2001). Many of the tegument polypeptides serve multiple functions at different steps in the viral replication cycle. For example, the tegument protein encoded by UL48 is the 65-kDa phosphoprotein VP16, a transcriptional activator of immediate-early gene products (Roizman and Knipe, 2001), and also a modulator of the UL41 gene product vhs (Lam et al., 1996), which degrades cellular and viral mRNA (Everly et al., 2002). Consistent with its location in the tegument, it has been shown that VP16 is required for proper viral assembly and egress (Mossman et al., 2000). Deletion of VP16 in HSV prevents the accumulation of enveloped cytoplasmic capsids. Though perinuclear enveloped virions do form, their envelope morphology appears altered (Mossman et al., 2000).

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Although it is generally agreed that HSV capsids acquire their final envelope by budding into a cytoplasmic organelle, little is known of the molecular details of this process. It seems reasonable to suppose that budding occurs as a result of interactions between the viral capsid, tegument proteins, and the cytoplasmic tails of envelope glycoproteins. This would ensure that all of the required components of the virus would come together to facilitate assembly of the mature infectious particle. Consistent with this hypothesis, several interactions have been identified between different components of tegument, and between tegument and glycoproteins. For example, in addition to its association with the tegument polypeptide vhs (Smibert et al., 1994; Lam et al., 1996), VP16 also binds the tegument protein VP22, the product of the UL49 gene (Elliott et al., 1995). Additionally, the HSV-1 glycoproteins gH, gB, and gD can be chemically crosslinked to VP16 in purified virions, suggesting that they are, at least, in very close proximity to this tegument component (Zhu and Courtney, 1994). In pseudorabies virus (PrV), the tegument proteins encoded by UL36 and UL37 have also been shown to associate with each other (Klupp et al., 2002). These proteins clearly play an important role in assembly, since deletion of either UL36 or UL37 in HSV-1 (Desai, 2000; Desai et al., 2001) and UL37 in PrV (Klupp et al., 2001) dramatically inhibit virus maturation. Furthermore, recent data have shown that the tails of the PrV envelope glycoproteins gE and gM bind to VP22 in a yeast two-hybrid study (Fuchs et al., 2002). Consistent with this observation, simultaneous deletion of gM and the gE/gI heterodimer results in reduced amounts of VP22 in the mature PrV particle, and in the formation of capsid-bound tegument aggregates in the cytoplasm (Brack et al., 1999, 2000; Fuchs et al., 2002). These studies also illustrate the considerable amount of redundancy present in the assembly process, as deletion of either gM or the gE/gI heterodimer alone had little effect on VP22 incorporation (Fuchs et al., 2002). Only simultaneous deletion of all three glycoproteins, or deletion of gM combined with deletion of the gE cytoplasmic tail, was sufficient to inhibit particle morphogenesis (Brack et al., 1999, 2000). Similarly, deletion of gD, gE, and gI in HSV-1 has recently been shown to cause a major defect in assembly, resulting in accumulation of unenveloped capsids in the cytoplasm that are embedded in tegument-like material. This result has been interpreted to imply that gD, gE, and gI act in a redundant fashion to anchor the virion envelope onto tegument-coated capsids (Farnsworth et al., 2003).

Crosslinking of VP16 to the cytoplasmic tails of gB, gH, and gD (Zhu and Courtney, 1994) provided the first indication of which tegument/glycoprotein tail interactions may occur in the HSV-1 particle. The potential association between VP16 and gH provides a particularly attractive model system for studying assembly, since gH has a short cytoplasmic tail consisting of 14 amino acid residues, making it easily amenable to mutagenic analysis. However, the fact that gH and the gH cytoplasmic tail can be deleted with no apparent effect on HSV assembly (Forrester et al., 1992; Wilson et al., 1994; Harman et al., 2002) suggests that, as in PrV, redundant protein–protein interactions might make a genetic analysis difficult. To circumvent this problem, we attempted to reconstitute VP16/gH association in a simplified, biochemically accessible system. To this end, we prepared Glutathione-*S*-Transferase (GST) fusion proteins that bear the cytoplasmic tail of gH or mutants thereof at their carboxy-termini. Following their binding to Glutathione-Sepharose beads and incubation with HSV-infected cell extracts, these fusion proteins were tested for their ability to interact with VP16. Here we report that the cytoplasmic tail of gH does indeed bind to the tegument protein VP16 in vitro, consistent with the in vivo studies of Zhu and Courtney (1994). Additionally, we have found that the interaction between the gH tail and VP16 in this experimental system is independent of the presence of other viral polypeptides. Furthermore, we have mapped the residues in the gH tail that are critical for binding VP16 and show that binding is optimal at physiological temperature. To test the significance of these findings for in vivo binding between VP16 and gH, we have transfected cells with constructs expressing green fluorescent protein (GFP) fused to wild-type or mutant versions of the gH cytoplasmic tail, and then infected the cells with HSV-1. By immunoprecipitating the extracts of these cells with anti-GFP antibodies, we have shown that VP16 and the gH carboxy-terminus interact with similar sequence dependence as in vitro.

Results

Generation of a GST-gH tail fusion to test for interaction with VP16

We used the vector pGEX-KG (Guan and Dixon, 1991) to prepare in-frame fusions between the coding sequence of GST and DNA encoding the cytoplasmic tail of gH (Fig. 1A). *Escherichia coli* were transformed with pGEX-KG or the gH tail-GST construct, treated with IPTG to induce expression, and subjected to SDS–PAGE. In Fig. 1B, Coomassie blue staining shows that fusion proteins of the correct size were readily apparent as the most prominent protein band in the gel. These bands were also reactive with anti-GST antibodies following immunoblotting (data not shown).

Binding of the tegument polypeptide VP16 to the gH tail fusion protein

We previously described methods for the isolation of HSV-containing organelles and putative assembly intermediates from the cytoplasm of HSV-infected cells (Harley et al., 2001). We reasoned that such organelles would be a convenient source of VP16 that may be capable of interacting with the HSV gH tail. To test this possibility, we

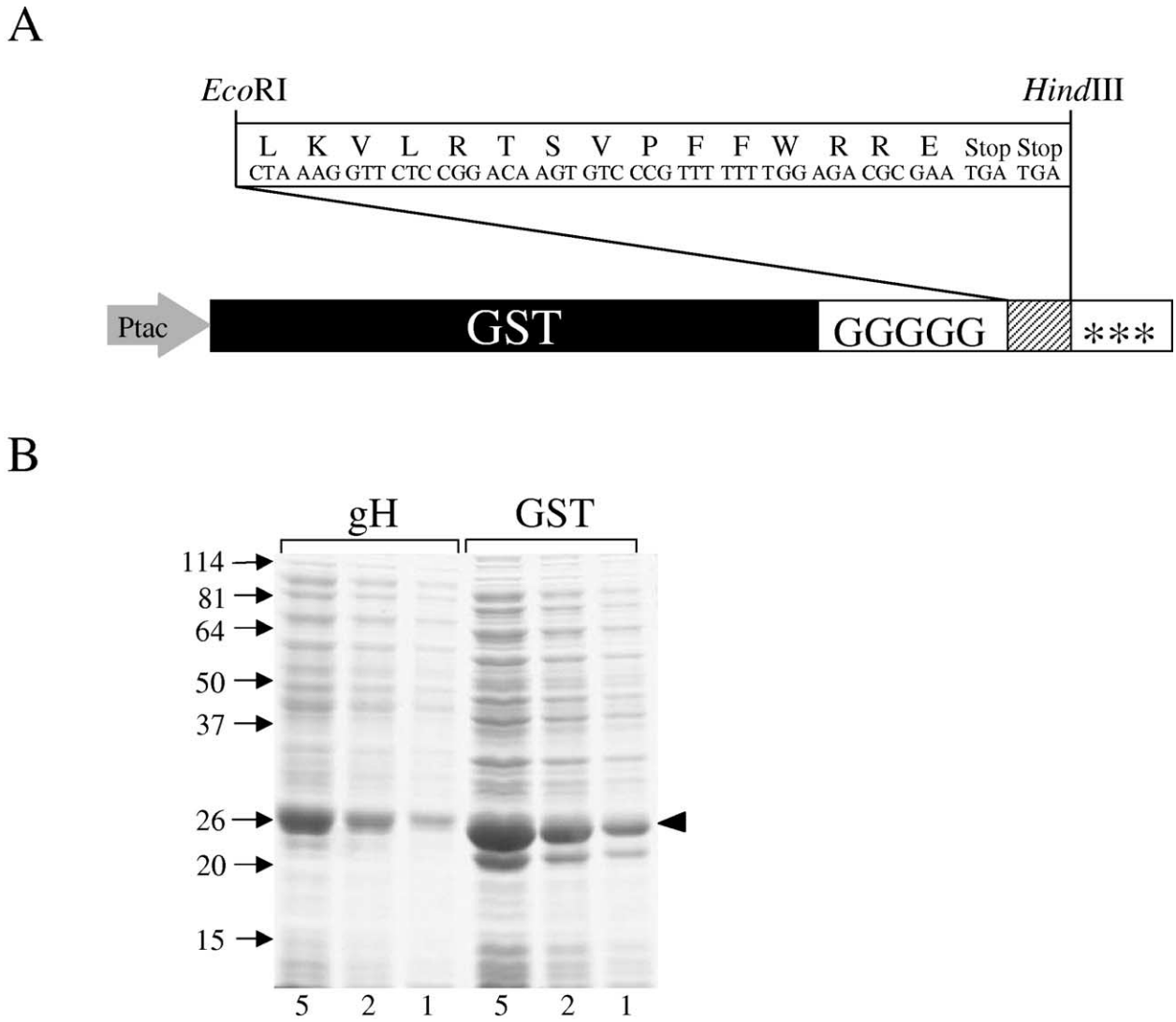

Fig. 1. Construction and expression of a GST-gH fusion protein. (A) Schematic of the fusion protein expression region of plasmid pGEX-KG. In the lower bar is indicated the IPTG inducible promoter Ptac, the direction of transcription (gray arrow), and the GST open reading frame (black bar). Following the polyglycine flexible hinge (GGGGG) is the polylinker region (hatched bar), flanked by the *Eco*RI and *Hind*III restriction sites used in this study. DNA encoding the gH cytoplasmic tail and the amino acid sequence of the tail are shown above the vector backbone. The tail is fused in-frame with GST at the *Eco*RI site and terminates with two in-frame stop codons prior to the *Hind*III site, as indicated. Asterisks indicate additional stop codons provided by the vector. Sequences required for bacterial growth and antibiotic selection are not shown. (B) *Escherichia coli* were transformed with the parental vector (GST) or with the plasmid encoding a fusion between GST and the gH tail (gH) as indicated. Following IPTG induction, 1, 2, or 5 volume equivalents (as indicated) of total bacterial extract were subjected to 12% SDS–PAGE and proteins were visualized with Coomassie brilliant blue. Positions and sizes (in kDa) of standard molecular weight markers are indicated at the left of the figure. Arrowhead at right indicates the position of GST and the GST-gH fusion.

prepared a postnuclear supernatant (PNS) from HSV-infected COS cells and solubilized the viral structural proteins by incubation with NP-40 and NaCl (we have previously found that many tegument polypeptides are insoluble in the PNS, perhaps due to their incorporation into assembling virions—data not shown). This detergent-solubilized extract was then incubated for 1 h with Glutathione-Sepharose beads, which were previously bound to equivalent amounts of GST or the gH tail-GST fusion protein (termed GST-gH). Material that was bound and unbound to the beads was resolved by SDS–PAGE and then analyzed by Western blot.

Fig. 2A shows our results for the binding of the tegument protein VP16 to GST-gH (gH); Western blots of material bound (b) and unbound (u) to the beads show the relative amount of VP16 binding to the gH tail under our conditions. Additionally, bead-bound material from the same experiment was subjected to SDS–PAGE and visualized by Coomassie staining to show the relative levels of GST and GST-gH fusion protein. To optimize our conditions, we tested binding at several different temperatures. We found that binding of VP16 to the gH tail was strikingly dependent on temperature (Fig. 2B). VP16 bound to GST-gH (gH) only at 37°C and failed to bind at 4°C or room temperature (RT) after a 1-h incubation.

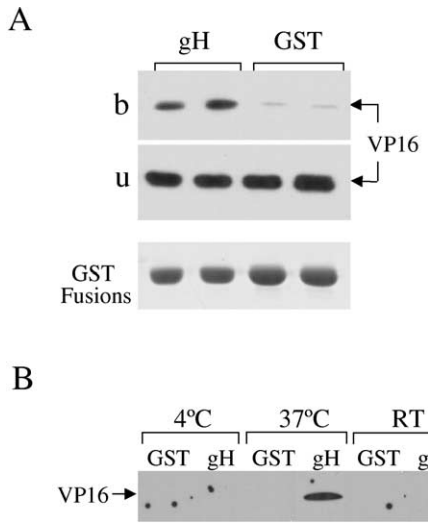

Fig. 2. Binding of the gH cytoplasmic tail to VP16. GST or GST-gH fusions were induced; bacterial extracts were prepared and equivalent amounts of each protein were bound to Glutathione–Sepharose beads. These beads were then incubated with cytosol prepared from HSV-1-infected COS cells at 37°C or as otherwise indicated. Bound or unbound polypeptides were resolved by 8% SDS–PAGE, transferred to a nitrocellulose filter, and probed with anti-VP16 antibody, as indicated. (A) Top two panels show anti-VP16 Western blots of duplicate samples bound (b) and unbound (u) to GST-gH (gH) and GST (as indicated above the figure). In the bottom panel, binding reactions were resolved by a 12% SDS–PAGE and stained with Coomassie brilliant blue to show the levels of GST fusion proteins used in this experiment. (B) Temperature dependence of VP16 binding. Infected cell cytosol was incubated with GST or GST-gH-coated Sepharose beads for 1 h at 4°C, 37°C, or room temperature (RT), as indicated at the top of the figure. Bound material was then Western blotted for VP16.

Lack of binding to GST, and restriction of binding to physiological temperature, was suggestive of a specific interaction between VP16 and the gH tail. However, this specificity was further examined in two different ways. First, we reasoned that if binding is specific, it should be saturable. To test this, constant, equal amounts of GST and GST-gH were incubated with increasing amounts of infected cell cytosol, and Western blotted for VP16 (Fig. 3A). Binding of VP16 to GST-gH (gH) saturated between 4 and 10 volume equivalents of cytosol, indicating that there are limited numbers of binding sites for VP16 within GST-gH, and therefore, VP16 binding to GST-gH is specific. Note that as increasing amounts of cytosol are added, levels of VP16 binding to GST-gH decrease. We speculate that this reproducible result may be caused by an increase in abundance of proteins, such as other tegument components, that may compete with VP16 for association with the gH tail when present at sufficient concentration. In contrast, binding of VP16 to GST continued to increase as increasing amounts of cytosol were added, indicating that the level of binding of VP16 to GST alone was not only low, but also nonsaturable, and presumably, nonspecific. As a second test for specificity, Fig. 3B shows the binding of GST and GST-gH to various cellular and viral polypeptides. The cellular protein Actin, the capsid scaffold proteins ICP35 and Pra (which share a common carboxy-terminal portion recognized by the antibody used), and the tegument protein VP22 all fail to bind to GST-gH under our conditions, indicating that gH does not nonspecifically bind abundant viral or cellular proteins.

Alanine scanning mutagenesis of the gH cytoplasmic tail

To identify residues in the gH tail that are important for VP16 binding, we performed alanine scanning mutagenesis, mutating either single residues or groups of residues in the gH carboxy-terminus. The mutants were generated in the same way as the wild-type gH tail-GST fusion (Materials and methods), and the resulting GST fusions named based on the residue(s) mutated to alanine. These mutants, as well as GST-gH and GST, were expressed and subjected to binding assays, and the relative levels of VP16 binding to the mutants were detected by Western blot. In addition, following SDS–PAGE of binding reactions, gels were Coo-

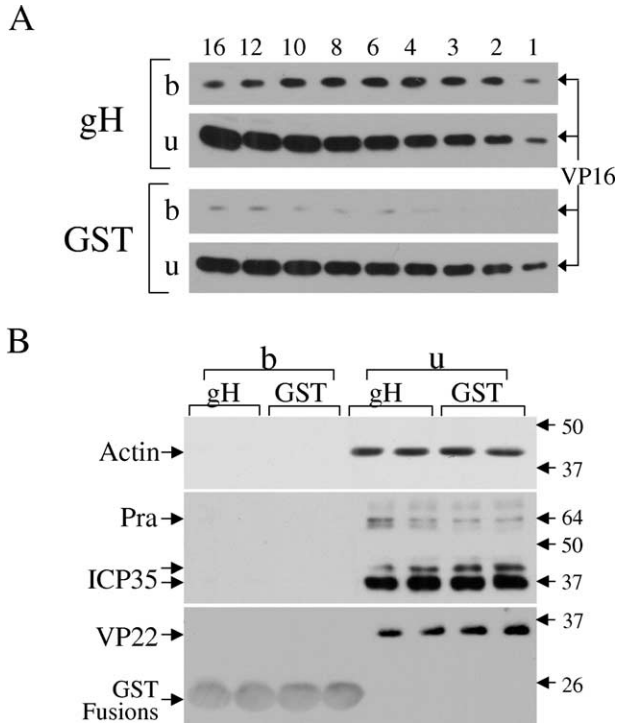

Fig. 3. Specificity of interaction between VP16 and the gH tail. (A) Saturation of VP16 binding to GST-gH. GST-gH- or GST-coated beads were incubated with increasing amounts of infected cell cytosol, and then Western blotted and probed for VP16. Panels show material bound (b) and unbound (u) to GST-gH (gH) or GST. Volume equivalents of cytosol added are indicated above the respective lanes. (B) Control cellular and viral proteins do not bind to the gH tail. Duplicate bindings of GST-gH (gH) and GST (as indicated above respective lanes) were subjected to Western blot and probed with antibodies specific to actin, ICP35 and Pra, or VP22 (as indicated to the left of the figure). Lanes representing bound (b) and unbound (u) material are indicated at the top of the figure. Position and sizes (in kDa) of molecular weight markers are indicated at the right.

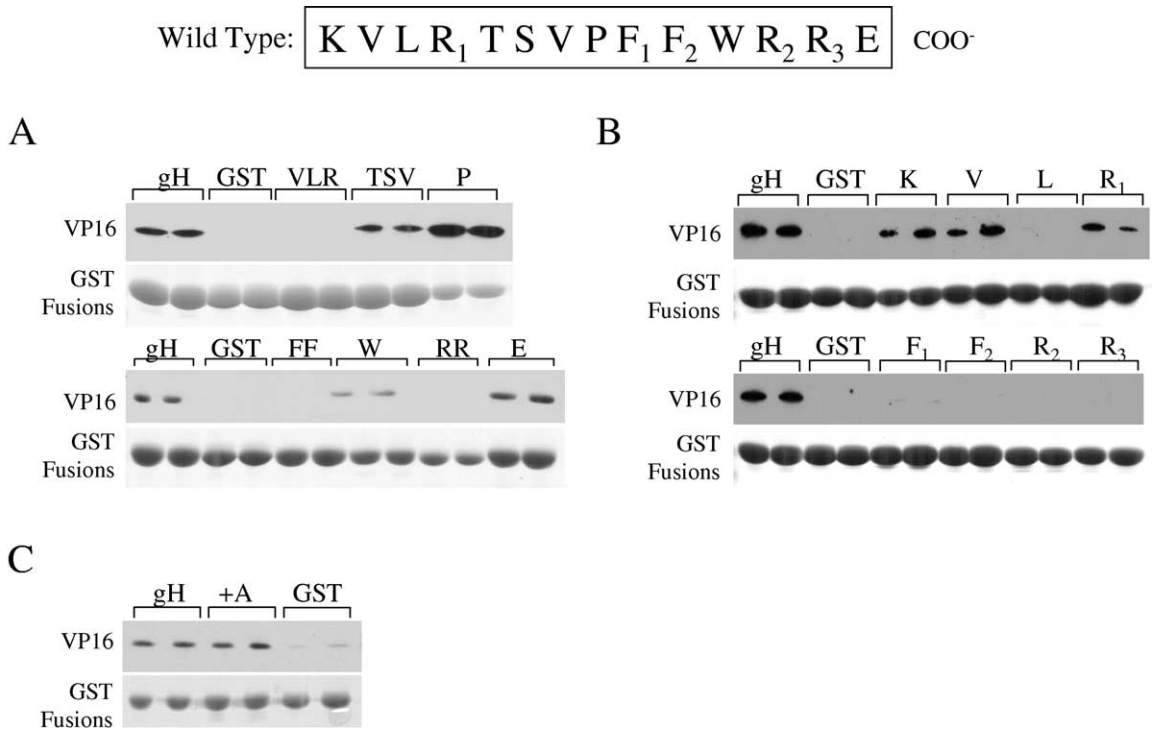

Fig. 4. Alanine scanning mutagenesis of the gH-tail. Single residues or groups of residues were mutated to alanine, and the resulting GST-gH mutants were named according to the residues changed. The mutants were subjected to binding experiments, in duplicate, and then Western blotted for VP16 or Coomassie stained for fusion protein levels, as indicated at the left of the panels. Binding of wild-type GST-gH (gH) and GST alone are shown as positive and negative controls. (A) Binding of VP16 to preliminary mutants of the gH tail, in which every residue in the gH tail was mutated alone or in groups to alanine. (B) Individual residues of the gH tail were mutated to alanine and fused to GST, and then subjected to a binding assay. (C) An extended gH tail mutant (+A) was constructed in which an extra alanine residue was added at the carboxy-terminus of the wild-type sequence. Wild-type gH tail sequence is indicated at the top of the figure.

massie-stained to show the relative levels of each of the fusion proteins present in the reaction. Fig. 4A demonstrates that mutation of the valine-leucine-arginine residues at the amino-terminal end of the tail (VLR), as well as mutation of the pair of centrally located phenylalanines (FF) and pair of arginines close to the carboxy-terminus (RR), abolish binding of VP16 to the gH tail, indicating that some or all of these residues play a critical role in VP16 binding. Mutating a carboxy-proximal tryptophan residue (W) to alanine consistently reduced binding to VP16, suggesting that it plays an important, albeit not crucial, role in VP16 binding under our conditions. Unexpectedly, mutation of the glutamic acid residue (E) at the C-terminal end of the tail, and the proline residue (P) at the center of the tail, actually enhanced binding of VP16 relative to the wild-type sequence. This result was especially apparent in the case of the P mutant. Each of the threonine-serine-valine residues amino-terminal to the center of the tail (TSV) appeared unimportant for binding, although the valine residue has been previously shown to play a role in modulating syncytium formation during infection (Wilson et al., 1994).

In light of the data above, we proceeded to make finer mutations in the gH tail by altering potentially important residues individually to alanine. Fig. 4B shows Western blots and Coomassie-stained gels of binding experiments using these new mutants. From these experiments we determined that the leucine residue (L) near the N-terminus of the tail, each of the central phenylalanine residues (F_1 and F_2), and each of the carboxy-proximal arginine residues (R_2 and R_3) are required for VP16 binding. The amino-proximal lysine (K), valine (V), and arginine (R_1) seem to play no role in VP16 binding.

Since the gH cytoplasmic tail is short, we were interested to know if the peptide inserts into a shallow groove on its binding partner. This is not without precedent—the sorting motif KDEL, present in the carboxy-termini of lumenal ER proteins, inserts into a short channel in its receptor, and addition of a single residue to the end of the motif abolishes binding (Munro and Pelham, 1987; Wilson et al., 1993). Therefore, we created a mutant with an extra alanine residue at the C-terminus of the gH tail and then tested its binding to VP16. As shown in Fig. 4C, the extended tail mutant (+A) bound to VP16 in a manner similar to wild-type.

Physiological temperature-dependent binding of VP16 to gH is controlled by the centrally located proline residue

We showed in Fig. 2B that VP16 binds to gH at 37°C after a 1-h incubation, but not at 4°C or at RT. Since mutating the proline residue in the gH tail to alanine dra-

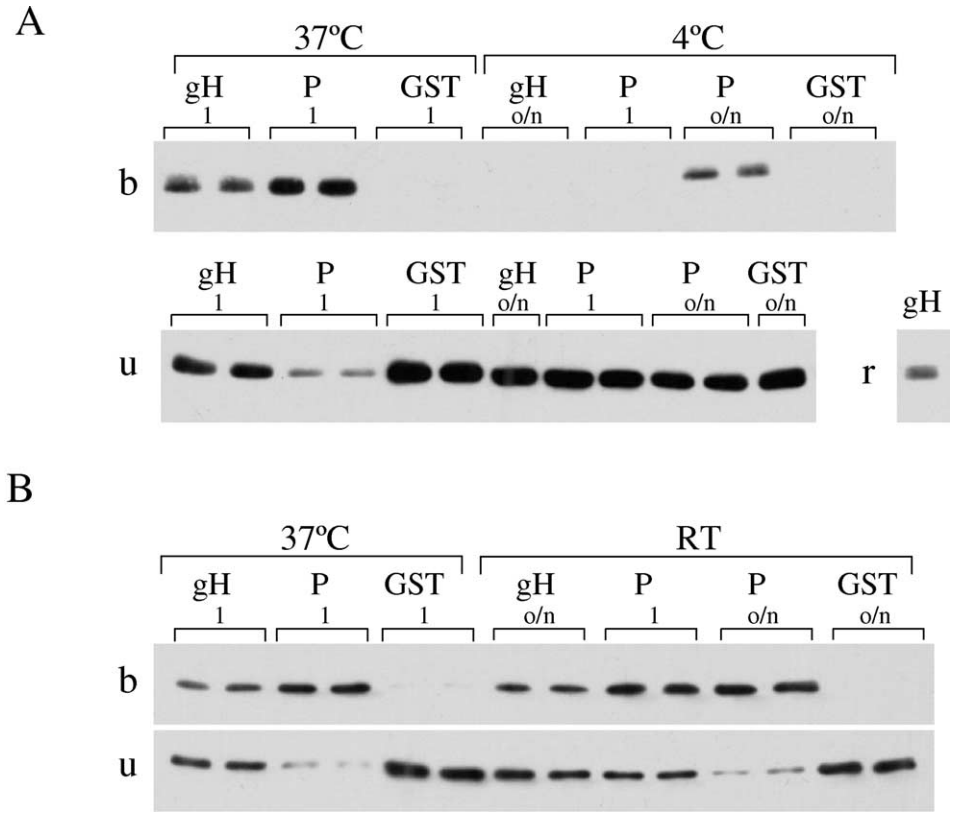

Fig. 5. Temperature dependence of VP16 binding to wt gH and the proline-to-alanine mutant. GST or the fusion proteins were incubated in duplicate with infected cell cytosol at either 4°C, RT, or 37°C, as indicated at the top of each figure. Binding took place for 1 h (1) or overnight (o/n), as indicated above each lane. Panels show Western blots for VP16 of either material bound (b) to the fusion proteins, or unbound (u). (A) Binding of VP16 to gH and P mutant compared at 4 and 37°C. Unbound material from one of the duplicate gH samples was then rebound (r) to fresh GST-gH-coated Sepharose beads for 1 h at 37°C. (B) Binding of VP16 to gH and P mutant compared at room temperature and 37°C.

matically increased binding of VP16 compared to wild-type under physiological conditions (Fig. 4A), we were interested to determine whether loss of the proline residue would alleviate the temperature restriction and enable binding of VP16 at lower temperatures. We therefore performed VP16 binding reactions with GST, GST-gH, and the P mutant at 37°C, 4°C, or RT for either 1 h or overnight (16–18 h). In Fig. 5, we show Western blots of bound (b) and unbound (u) material from these experiments. Fig. 5A demonstrates that mutation of the central proline residue to alanine does result in the ability of this tail to bind VP16 at 4°C. However, binding must be for an extended period of time, 16–18 h. The wild-type tail continues to fail to interact with VP16 at 4°C even after this extended incubation. Failure of VP16 to associate with the wild-type gH tail even after prolonged incubation at 4°C is not due to denaturation or other means of inactivation of VP16, since when cytosol that had been incubated overnight at 4°C with the gH tail was warmed to 37°C and reincubated with fresh GST-gH coated beads, the VP16 in the extract was able to bind (indicated by "r" in Fig. 5A).

Fig. 5B shows binding of GST, GST-gH, and the proline mutant at room temperature compared to 37°C. In contrast to our results at 4°C, VP16 was capable of binding to the wild-type gH tail after an overnight incubation. Furthermore, the proline mutant once again demonstrated increased VP16 binding, as it only required 1 h to bind a significant level of VP16, whereas wild-type gH was incapable of binding detectable levels of VP16 in 1 h at room temperature (Fig. 2B).

VP16 binds to the gH tail independently of other viral polypeptides

Since VP16 is part of the complex network of proteins in the HSV tegument, we were interested to determine whether VP16 binding to the gH cytoplasmic tail was mediated by other viral polypeptides. To test this, we used a rabbit reticulocyte lysate system to express ^{35}S-labeled VP16 polypeptide in vitro, in the absence of any other viral factors. We then incubated the in vitro translated (IVT) VP16 with Glutathione-Sepharose beads, which had been precoated with GST, GST-gH, or various mutants. Fig. 6A shows binding of IVT VP16 under standard conditions (incubation at 37°C for 1 h) with GST, GST-gH, as well as two mutants, P, which enhances binding of cytosolic VP16

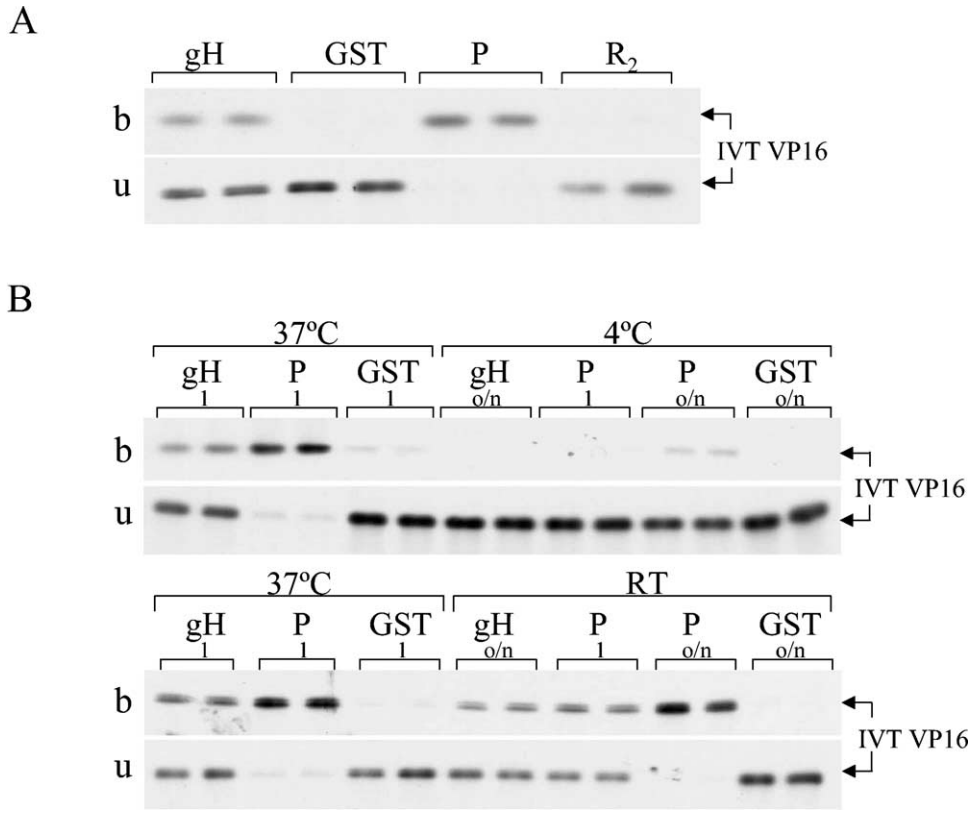

Fig. 6. Binding of in vitro translated (IVT) VP16 to the gH tail. ^{35}S-labeled VP16 was expressed by in vitro translation and incubated with GST-gH, GST, or various GST-gH-derived mutants as indicated in the figure. Shown are autoradiographs of material bound (b) and unbound (u) to the GST fusions, as indicated at the left of the figure. (A) Binding of IVT VP16 to GST-gH, GST, as well as the P and R_2 mutants, as indicated. (B) IVT VP16 was incubated with gH, GST, and the P mutant at 37°C, 4°C, or RT, as in Fig. 5.

compared to wild-type (Fig. 4A); and R_2, which diminishes binding of cytosol-derived VP16 (Fig. 4B). We found that IVT VP16 bound to GST-gH but not to GST alone and that it bound to the two mutants in a similar fashion as cytosolic VP16 (Fig. 6A). To further compare the binding characteristics of IVT and cytosolic VP16, we tested the temperature dependence of binding. In Fig. 6B, autoradiographs of IVT VP16 binding to GST-gH, GST, and the P mutant show results similar to those obtained using cytosol-derived VP16: IVT VP16 does not bind GST-gH at 4°C after an overnight incubation, whereas the P mutant binds. Furthermore, IVT VP16 binds GST-gH after an overnight incubation at RT, whereas it binds to the P mutant after both an overnight and a 1-h incubation. Since there was no apparent difference between cytosolic and IVT VP16 with respect to VP16/gH tail interaction, we conclude that VP16 binds to the gH cytoplasmic tail independently of other viral polypeptides.

VP16 binds to the gH cytoplasmic tail in vivo

All of the experiments performed above utilized in vitro techniques to establish the binding of VP16 to gH. To ascertain that a similar interaction occurs in vivo, immunoprecipitation experiments were carried out in infected cells. To do this, we fused the gH tail to the carboxy-terminus of GFP. Details of the construction of this plasmid, as well as plasmids encoding GFP fused to gH tail mutants, are described under Materials and methods.

COS cells were mock transfected or transfected with GFP alone or GFP-gH fusions. Approximately 30 h post-transfection, the cells were infected with HSV. Fifteen hours postinfection the cells were collected and extracts were prepared (see Materials and methods). The cell lysates were then precleared with Protein A agarose beads at the temperature later used for immunoprecipitation to minimize subsequent nonspecific binding. Precleared lysates were then incubated for 30 min at 30 or 37°C with Protein A agarose beads which had been precoated with anti-GFP polyclonal antibodies. After washing the beads, immunoprecipitated material, as well as material that did not bind to the beads, was subjected to SDS–PAGE and Western blotted with anti-GFP or anti-VP16 antibodies to assess the efficiencies of GFP immunoprecipitation and to test whether VP16 had been coimmunoprecipitated. Fig. 7A shows that VP16 was coimmunoprecipitated with the GFP-gH fusion, but not with GFP alone or with mock-transfected cells. Additionally, VP16 did not coimmunoprecipitate with

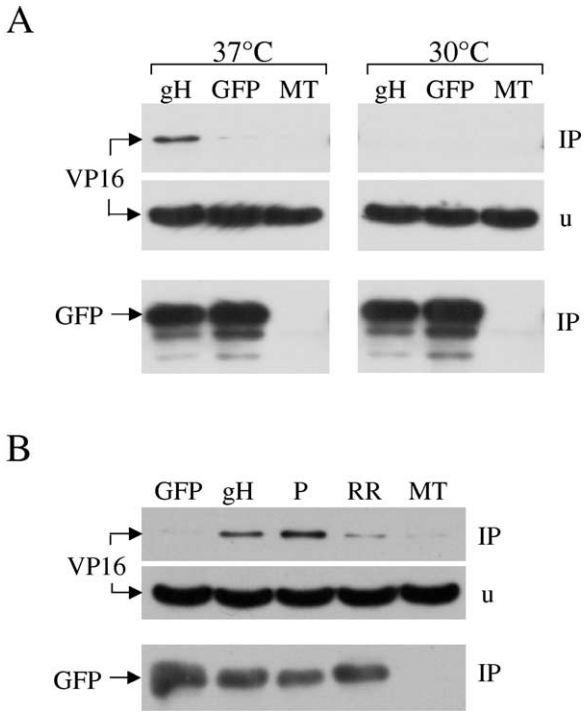

Fig. 7. Coimmunoprecipitation of GFP-gH and VP16. COS cells were mock-transfected (MT) or transfected with plasmids expressing GFP alone, or GFP fused to the gH tail (gH) or mutants thereof as indicated, and then infected with HSV-1. Fifteen hours postinfection, the cells were incubated with lysis buffer (see Materials and methods) for 30 min on ice. After pelleting the debris, cell extracts were incubated with Protein A agarose beads precoated with rabbit anti-GFP antibody for 30 min at the indicated temperatures and washed in lysis buffer. Material that immunoprecipitated with anti-GFP antibody (IP), as well as material that was in the unbound fraction (u), was subjected to SDS–PAGE and Western blotted using mouse anti-VP16 or anti-GFP antibodies, as indicated at the left of the panels. Note that 1/20th of the total unbound material was run on the gel. (A) Coimmunoprecipitation of VP16 at 37 or 30°C by wild-type gH tail. (B) Coimmunoprecipitation of VP16 by gH tail mutants at 37°C. P mutant is used as an example of a mutant that enhances binding to VP16, and RR represents a mutant that abolishes binding to VP16 in vitro (see Fig. 4A). Note that 1/10th of the total unbound material was loaded on the gel.

GFP-gH at 30°C (Fig. 7A) or at 4°C (data not shown), consistent with our in vitro binding experiments. In the lower panel, blotting with anti-GFP antibodies shows that similar amounts of GFP and GFP-gH were present in the immunoprecipitate.

To test the correspondence between in vivo and in vitro binding, we assessed the in vivo binding characteristics of some of the mutants generated in our in vitro investigation. To this end, we prepared GFP fusions to two gH tail mutants, the proline-to-alanine (P) mutant, which binds with greater efficiency to VP16 (Fig. 4A), and the double arginine-to-alanine (RR) mutant, which does not bind VP16 in vitro (Fig. 4A). Fig. 7B shows the results of immunoprecipitation studies using these mutants. Consistent with our in vitro studies, the P mutant showed enhanced binding to VP16 compared to wild-type GFP-gH, even though it was present at reduced levels in the immunoprecipitate (see Western blot using anti-GFP antibody in bottom panel). Furthermore, the RR mutant showed diminished binding to VP16. Note that in Figs. 7A and B, the amount of unbound material loaded on the gels was 20- and 10-fold less (respectively) than the amount of immunoprecipitated material, and therefore only a very small proportion of total VP16 was observed to coimmunoprecipitate with the GFP-gH fusion. This may reflect the fact that, during HSV infection, only a small fraction of the structural proteins synthesized are assembled into virions. Additionally, the GFP proteins are only expressed in transfected cells, which are in a minority, whereas all of the cells are infected with HSV, and thus express VP16.

Discussion

Zhu and Courtney reported that gH could be crosslinked to VP16 in purified virions (Zhu and Courtney, 1994), indicating a potential interaction between the two proteins. We have examined this possibility by preparing a gH tail-GST fusion protein and incubating it with HSV-infected cell cytosol as a source of VP16. Under these in vitro conditions, we have shown that the tegument protein VP16 does indeed bind to the cytoplasmic domain of gH. Under our conditions, the binding of VP16 to the gH C-terminus is saturable, indicating that there are a limited number of VP16 binding sites on the fusion protein. The gH tail does not interact with HSV structural proteins VP22 and ICP35, nor with the cellular protein, actin. Additionally, we have found that VP16 binds to the gH cytoplasmic tail in a strikingly temperature-dependent manner; binding within 1 h occurs only at 37°C, but not at 4°C or room temperature. Taken together, we believe that these data suggest that the in vitro interaction of VP16 with the gH cytoplasmic tail is specific.

By performing alanine scanning mutagenesis of the gH tail, we have identified five residues that are critical for VP16 binding. The five residues are interspersed in the tail, with a leucine residue (L) at the N-terminal portion, two paired phenylalanine residues (FF) in the center, and two paired arginine residues (RR) toward the carboxy end. Unexpectedly, we also found that mutation of the C-terminal glutamic acid residue (E), and the central proline residue (P), actually increased the amount of VP16 bound; enhanced binding of VP16 to the proline mutant was particularly apparent.

There is no extensive conservation of the sequence of the gH cytoplasmic tail among different species of herpesviruses. However, inspection of predicted gH tail sequences deposited in the Swissprot, PIR, and translated GenBank databases reveals that two residues, which appear in this study to play an important role in VP16 interaction, are somewhat conserved among the alphaherpesviruses. The leucine residue at the third position C-terminal from the transmembrane domain is conserved among bovine herpes-

virus (BHV) type 1, BHV-2, BHV-5, equine herpesvirus (EHV) type 1, EHV-4, PrV, feline herpesvirus (FeHV) type 1, and canine herpesvirus (CaHV) type 1, although it is not conserved in HSV-2 (where it is instead a valine), nor in varicella zoster virus (VZV). The proline eight residues C-terminal from the transmembrane domain, in the center of the tail, is also conserved in several different species of herpesviruses, including HSV-2, BHV-1, BHV-2, and BHV-5, CeHV-1, and FeHV-1; in VZV the proline residue is predicted to be one residue further from the inner surface of the envelope.

Binding of VP16 to gH was strikingly affected by temperature. This could indicate some temperature-dependent biochemical modification of VP16, or of other polypeptides in the extract, which may be a prerequisite for interaction with the gH tail. However, preincubation of infected cell extracts at 37°C did not permit subsequent binding of VP16 to GST-gH at 4°C (data not shown), suggesting that if such modifications occur, they are reversed at 4°C or require the simultaneous presence of the gH tail. Alternatively, the temperature dependence of binding of VP16 to gH, together with the enhanced binding of VP16 to the proline mutant and the distribution of residues involved in VP16 association, suggested to us that VP16/gH interactions might be dependent on the structural conformation of the gH tail. In this model, the conformation of gH at 37°C is compatible with VP16 binding, but at reduced temperatures the tail undergoes conformational changes which preclude its association with VP16. Since the proline-to-alanine mutant is capable of binding VP16 at room temperature after 1 h, and at 4°C after an overnight incubation, loss of the proline may favor the tail adopting a binding conformation even below 37°C. We are investigating the role of the structural conformation of the gH tail in VP16 association using 1D and 2D NMR studies at various temperatures.

Since VP16 is part of the complex tegument structure, and a number of tegument polypeptides are known to interact with VP16, we were interested to test whether VP16 bound to the gH tail directly or via another viral protein. We therefore translated VP16 in an in vitro system and studied its binding to gH. We found that in vitro translated VP16 does bind specifically to GST-gH and that the temperature dependence of binding and dependence on the presence of particular residues in the gH tail are similar to that of infected cell VP16. This leads us to conclude that, under our conditions, binding of VP16 to the gH tail is independent of other viral polypeptides.

Since the experiments discussed above were all performed under in vitro conditions, we wanted to confirm that this interaction also occurs in vivo. To test this, we conducted coimmunoprecipitation experiments using the gH tail fused to GFP. In this fashion we were able to demonstrate that VP16 can interact specifically with the gH tail in an infected cell. Additionally, by performing these experiments using GFP fused to mutated gH tails, we have shown that at least two of the mutants behave with similar characteristics in vivo as in vitro. We also found that the in vivo association between VP16 and the gH carboxy-terminus is dependent on temperature, binding at 37°C, but not at lower temperatures, consistent with our in vitro data.

The association between VP16 and gH may be one of many interactions between glycoprotein tails and tegument proteins that facilitate the budding of capsids into organelles and the incorporation of tegument and/or glycoproteins into enveloping virions. When VP16 is deleted, cytoplasmic capsids fail to envelope, and perinuclear virions accumulate that appear to have a much smoother envelope than wild-type particles (Mossman et al., 2000). This may imply that VP16 could play a key role in sequestering membrane proteins to the site of envelope formation. However, a recent study appears to indicate that the C-terminal domain of gH is dispensable for gH incorporation into mature, secreted virions (Harman et al., 2002), which would suggest that gH tail/tegument interactions are not essential for incorporation of gH into mature particles. This is consistent with the observation that deletion of the nine terminal amino acids of gH does not significantly reduce PFU yield, although there are effects upon syncytium formation and the rate of entry of virions during an infection (Wilson et al., 1994; Browne et al., 1996b). It is possible that there are additional redundant glycoprotein–glycoprotein interactions in the envelope that facilitate incorporation of gH. In this regard it is interesting to note that even though the tails of PrV gE and gM interact with VP22, these glycoproteins nevertheless can become virion associated even when VP22 is absent (Fuchs et al., 2002). Similarly, it would appear that VP16 is not dependent upon the tail of gH to ensure its own incorporation into virions, since the retargeting of gH to locations not utilized for HSV envelopment apparently does not affect VP16 incorporation (Browne et al., 1996a). Presumably, VP16 interacts redundantly with other tegument proteins (Smibert et al., 1994; Elliott et al., 1995) or glycoproteins such as gB and gD (Zhu and Courtney, 1994) to mediate its incorporation into the particle in the absence of gH. It seems that, as in PrV, deletion of several glycoprotein tails and/or tegument proteins will be required to affect incorporation of tegument proteins or glycoproteins into the assembling particle.

Materials and methods

Cells and viruses

COS7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 1% penicillin-streptomycin and 10% fetal calf serum (Gibco Laboratories). HSV strain SC16 was prepared as previously described (Church and Wilson, 1997).

Antibodies

The anti-VP16 14-5 monoclonal antibody was obtained from Santa Cruz Biotechnology. The polyclonal, rabbit antiserum raised against the HSV tegument protein VP22 was kindly provided by Dr. John Blaho. Anti-actin rabbit antiserum was obtained from Sigma (A-2066). The rabbit anti-ICP35 antibody MCA406 was purchased from Serotech. Monoclonal anti-GFP mouse antibody was acquired from Clontech (Catalogue no. 8362-1). Polyclonal anti-GFP rabbit antibody (ab-290) was purchased from Novus Biological.

Construction of gH tail fusion plasmids

The predicted cytoplasmic tail of HSV glycoprotein gH was fused to the carboxy-terminus of GST using the GST vector pGEX-KG (Guan and Dixon, 1991). For the wild-type tail of gH, the complementary oligonucleotides 5′AATTCTAAAGGTTCTCCGGACAAGTGTCCCGTTT-TTTTGGAGACGCGAATGATGAAC TAGTTA3′ and 5′ AGCTTAACTAGTTCATCATTCGCGTCTCCAAAAAA-ACGGGACACTTGTCCGGAGAACCTTTAG3′ were synthesized and annealed. This generates a dsDNA fragment with *Eco*RI and *Hind*III ends, and which encodes the gH cytoplasmic tail followed by two stop codons. Ligation of this fragment with *Hind*III/*Eco*RI-digested pGEX-KG DNA results in an in-frame fusion of the GST and gH tail coding regions at the *Eco*RI site. Mutants of the gH cytoplasmic tail were generated in a similar fashion, with appropriate codons or groups of codons mutated to encode alanine. Each mutant also encoded its own unique restriction site between the second stop codon and the *Hind*III site, to facilitate identification by restriction analysis. In many of the mutant oligonucleotides, the sequence encoding the two phenylalanines at positions 9 and 10 of the tail was changed from 5′TTTTTT3′ to 5′TTATTT3′ to facilitate sequencing. Additionally, the mutant sequences differ from the wild-type oligonucleotides in that they lack a T on the 5′-3′ primer (second base from the 3′ end) and an A on the 3′-5′ primer (seventh base from the 5′ end), immediately adjacent to the *Hind*III site.

A series of plasmids was constructed expressing the gH cytoplasmic tail or mutants thereof fused to the carboxy-terminus of GFP, using a similar method to the above. To construct the wild-type gH tail, the complementary oligonucleotides, 5′ AATTCTAAAGGTTCTCCGGACAAGTGTC-CCGTTCTTTTGGAGACGCGAATGATGATGCGCAG 3′ and 3′ GATTTCCAAGAGGCCTGTTCACAGGGCAA-GAAAACCTCTGCGCTTACTACTACGCGTCCTAG 5′, were annealed and ligated to pEGFP-C3 vector (BD Biosciences Clontech) that was previously digested with *Eco*RI and *Bam*HI. This generates a plasmid encoding a carboxy-terminal fusion of the gH tail to GFP. To construct GFP fusions carrying mutations in the proline and paired arginine residues, oligonucleotides were synthesized in which the appropriate codon(s) were changed to those of alanine.

Expression of GST fusion proteins and binding of fusions to Glutathione-Sepharose beads

All bacterial growth was at 37°C. Appropriate plasmids were transformed into the *E. coli* GST fusion protein expression strain BL21 (Stratagene) and overnight stationary phase cultures were prepared. These were used to inoculate fresh medium to an OD_{600} of 0.2, and the cultures were grown with vigorous shaking to an OD_{600} of 0.9. At this point, fusion protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 1 mM, and cells were grown for a further 3 h. The bacteria were then centrifuged at 8000 rpm for 8 min at 4°C in a GS-3 rotor; the media were removed, and pellets were frozen at −20°C overnight. The pellets were then thawed, resuspended in lysis buffer [PBS, 0.05% Tween 20, 50 mM EDTA, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml antipain, 1 mg/ml lysozyme], and sonicated using a probe sonicator. Debris was pelleted at 10,000 rpm for 10 min at 4°C in a SS-34 rotor, and the resulting supernatant was incubated with Glutathione-Sepharose beads (Pharmacia), which had been previously washed twice in PBS. Following overnight incubation at 4°C, the beads were washed twice with Buffer 1 [50 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.5% Triton X-100], twice with Buffer 2 [50 mM Tris–HCl pH 7.6, 1.3 M NaCl, 0.5% Triton X-100], and then twice again with Buffer 1. The resulting beads, bearing specifically bound GST or GST fusion proteins, were then incubated with HSV-infected cell extracts, prepared as described below.

Preparation of HSV-1 infected cell extracts and binding assays

Confluent monolayers of COS-7 cells were infected with HSV-1 strain SC16 at a multiplicity of 3 and incubated for 16–18 h at 37°C, and a PNS was prepared as previously described (Harley et al., 2001).

To test for the binding of tegument polypeptides to GST fusion proteins, PNS was adjusted to 150 mM NaCl, 0.5% NP-40, 5 μg/ml leupeptin, 5 μg/ml antipain, 1 mM PMSF, 25 mM Tris–HCl pH 7.6, 100 mM sucrose, 1 mM $MgCl_2$, and then incubated for 1 h on ice to ensure organelle solubilization and release of organelle-associated tegument polypeptides. The mixture was then centrifuged at 53,000 rpm for 1 h in a TLA 100.3 rotor, and the resulting supernatant was incubated at various temperatures and times with Sepharose-bound GST fusion proteins. At the end of the incubation period, the beads were recovered, washed five times in Buffer 1 (see above), and bead-bound and unbound

material were examined following SDS– PAGE and Western blotting, as previously described (Harley et al., 2001).

Production of radiolabeled in vitro translated VP16

A plasmid encoding the VP16 open reading frame under the control of the T7 promoter was transcribed using the TNT Rabbit Reticulocyte Lysate in vitro translation system (Promega), as per the manufacturer's protocol. Redivue ^{35}S-labeled methionine (Amersham Pharmacia) was added to the in vitro translation reaction to generate labeled VP16. The IVT VP16 was then mixed with PBS and 5 μg/ml leupeptin, 5 μg/ml antipain and incubated at various temperatures and times with GST fusion protein-coated Glutathione-Sepharose beads, as described above. Materials that were bound and unbound to the beads were then subjected to SDS–PAGE, and the gel was treated in 30% MeOH, 10% AcOH for 30 min, Enhance (NEN Life Sciences Product, Inc.) for 1 h, and 1% glycerol for 30 min, and then dried using a Bio-Rad Gel Drier. The dried gel was then exposed to film at −70°C, and the autoradiograph was developed.

Coimmunoprecipitation of GFP-gH and VP16

COS cells were mock-transfected or transfected with plasmids expressing GFP alone, or GFP fused to the gH tail. Approximately 30 h posttransfection, the cells were infected with HSV-1 (strain SC16) at an m.o.i. of 10. After 15 h, the cells were washed with PBS and incubated with lysis buffer [20 mM Tris–Cl pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 0.5 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml antipain, 200 μg/ml BSA] for 30 min on ice. The extracts were then pelleted to remove debris, and the resulting cell lysates were precleared by incubating for 20 min at the immunoprecipitating temperatures (37 or 30°C) with Protein A agarose beads (Sigma) that had been prewashed twice in lysis buffer. The supernatants were then mixed with fresh Protein A agarose beads that had been preincubated at 4°C for 1 h with 10 μg of polyclonal anti-GFP antibody. Incubation was then performed for 30 min at indicated temperatures. The agarose beads were then washed five times in lysis buffer; then bead-bound and unbound material were subjected to SDS–PAGE and Western blotted using mouse anti-VP16 or anti-GFP monoclonal antibodies.

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